Follistatin attenuates early liver fibrosis: effects on hepatic stellate cell activation and hepatocyte apoptosis

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Patella, Shane, David J. Phillips, Jorge Tchongue, David M. de Kretser, and William Sievert. Follistatin attenuates early liver fibrosis: effects on hepatic stellate cell activation and hepatocyte apoptosis. *Am J Physiol Gastrointest Liver Physiol* 290: G137–G144, 2006. First published August 25, 2005; doi:10.1152/ajpgi.00080.2005.—Activin A, a member of the transforming growth factor-β superfamily, is constitutively expressed in hepatocytes and regulates liver mass through tonic inhibition of hepatocyte DNA synthesis. Follistatin is the main biological inhibitor of activin bioactivity. These molecules may be involved in hepatic fibrogenesis, although defined roles remain unclear. We studied activin and follistatin gene and protein expression in cultured rat hepatic stellate cells (HSCs) and in rats given CCl_4 for 8 wk and examined the effect of follistatin administration on the development of hepatic fibrosis. In activated HSCs, activin mRNA was upregulated with high expression levels, whereas follistatin mRNA expression was unchanged from baseline. Activin A expression in normal lobular hepatocytes redistributed to periseptal hepatocytes and smooth muscle actin-positive HSCs in the fibrotic liver. A 72% reduction in fibrosis, maximal at *week 4*, occurred in CCl_4-exposed rats treated with follistatin. Hepatocyte apoptosis decreased by 87% and was maximal at *week 4* during follistatin treatment. In conclusion, activin is produced by activated HSCs in vitro and in vivo. Absence of simultaneous upregulation of follistatin gene expression in HSCs suggests that HSC-derived activin is biologically active and unopposed by follistatin. Our in vivo and in vitro results demonstrate that activin-mediated events contribute to hepatic fibrogenesis and that follistatin attenuates early events in fibrogenesis by constraining HSC proliferation and inhibiting hepatocyte apoptosis.

activin; cirrhosis; hepatocytes

PROGRESSIVE HEPATIC FIBROSIS is characterized by excess deposition of extracellular matrix components after the activation of resident fibroblastic cells, such as portal tract fibroblasts, and transformation of hepatic stellate cells (HSCs) into collagen-producing myofibroblasts (1, 7, 31). Ample data support the role of transforming growth factor (TGF)-β as a major profibrogenic cytokine in cutaneous wound healing and in pathological fibrotic processes in the kidney, lung, and liver (3, 8, 11, 26). TGF-β is overexpressed during experimental liver fibrosis and is a potent regulator of HSC activation (11). Recent reports have demonstrated that the development of liver fibrosis can be attenuated by blockade of TGF-β signaling (10, 29). However, other data suggest that TGF-β is not the sole mediator of fibrosis. For example, significant overexpression of TGF-β in transgenic animals leads to only moderate degrees of fibrosis (1). Furthermore, attenuation of TGF-β signaling in animal models of hepatic fibrosis ameliorates but does not completely abrogate fibrosis (10, 29), suggesting that other factors contribute to this process.

We (25) have previously demonstrated that serum levels of activin A are significantly elevated in subjects with chronic viral hepatitis, whereas serum follistatin levels remain normal. Furthermore, hepatitis B virus replication is associated with a pronounced dysregulation in the normal activin-follistatin axis. Activin bioactivity is regulated by follistatin, an extracellular binding protein that is a potent neutralizer of activin action (27). In the normal liver, activin A is constitutively expressed by hepatocytes and acts as an autocrine inhibitor of hepatocyte DNA synthesis (39). Activin induces hepatocyte apoptosis in vitro and in vivo (12, 33) and regulates liver mass (13, 21). After partial hepatectomy, follistatin infusion increases hepatocyte regeneration (19, 20) by blocking activin inhibition of DNA synthesis (27) and profoundly alters liver mass. Several lines of evidence support a role for activin A in hepatic fibrosis. Activin-$β_A$ mRNA increases significantly in hepatic fibrosis (34), and activin A localization in the fibrotic liver redistributes from the hepatocyte to HSC (5). Until now, the potential for follistatin to diminish hepatic fibrosis has not been examined.

In the present study, we characterized the gene and protein expression of activin A and follistatin in cultured HSCs. We demonstrate that HSCs secrete activin during transformation to myofibroblasts and, during in vivo studies in the fibrotic liver, that activin and follistatin gene expression is altered and that activated HSCs express activin. Finally, follistatin administration attenuates hepatocyte apoptosis and the development of early hepatic fibrosis in CCl_4-treated animals.

METHODS

Animal model and experimental design. The Monash Medical Centre Animal Ethics Committee approved this project. Cirrhosis was induced in male Wistar rats by an intraperitoneal injection of CCl_4 and olive oil at a final concentration of 0.4 mg/kg three times weekly for 8 wk. Control animals received olive oil alone. Groups of five animals were killed at 2, 4, and 8 wk, and whole livers were removed for analysis.

Follistatin administration. In a separate group of animals, 2 μg of recombinant human follistatin 288 (rh-FS288) were injected intravenously every second day during concurrent CCl_4 administration. Animals were killed at 2, 4, and 8 wk.

Determination of hepatic hydroxyproline content. Liver hydroxyproline content was quantified as previously described (16). Briefly, frozen liver samples were hydrolyzed in 6 N HCl at 110°C for

16–18 h in Teflon-coated tubes. Samples were cooled, 40 mg Dowex (Ajax Chemicals; Sydney, NSW, Australia) was added to each sample, and samples were vortexed. The hydrolate was filtered into fresh tubes, and sample pH was adjusted to 7.4. Total hydroxyproline content was measured against standard preparations of hydroxyproline in the range of 5.0–0.156 μg. The inter- and intra-assay variability was 3% and 6%, respectively.

Activin A quantification. Activin A was measured by a two-step sandwich ELISA using human recombinant activin A as previously described (18). The mean intra- and inter-assay coefficients of variation were less than 10% and 14%, respectively, and the minimum detection limit was 0.08 pg/ml.

Histology and immunohistochemistry. For immunohistochemical examination, 4-μm formalin-fixed paraffin-embedded tissue sections were used. Deparaffinized slides underwent microwave heat retrieval in 0.1 M sodium citrate buffer (pH 7.4) when required. 3',3-Diaminobenzadine was used in all staining procedures, and sections were counterstained with hematoxylin. Negative control sections substituted the primary antibody for an irrelevant antibody (normal mouse Ig) in all procedures. Sirius red-stained biopsies were staged using the modified Knodell score (15).

To localize activin A, a monoclonal antibody to the activin β_A-subunit was applied at a final concentration of 18 μg/ml and incubated overnight at 4°C. Reactivity was amplified using a CSA signal amplification kit (DAKO; Carpenteria, CA) according to the manufacturer's instructions. A monoclonal antibody against human follistatin 288 (1:150) was used to determine immunoreactivity (kindly provided by Professor Nigel Groome, Oxford Brookes University) (23). The staining procedure was conducted as for the activin β_A-subunit. α-Smooth muscle actin (α-SMA; 1:100, DAKO) was used to detect activated HSCs.

Hepatocyte apoptosis was assessed by TdT-mediated dUTP nick-end labeling (TUNEL) following the manufacturer's instructions (ApopTag Plus, Intergen). Sections were counterstained with hematoxylin, and apoptotic cells were identified by morphology and counted at ×400 magnification. Results were expressed as the number of apoptotic cells per millimeter squared of tissue.

Confocal microscopy. HSC coexpressing activin A and α-SMA were detected using confocal fluorescent microscopy. Dewaxed sections were incubated with the biotinylated activin A monoclonal antibody (25 μg/ml, Oxford Bio-Innovations; Oxford, UK) for 1 h at room temperature. After being washed, sections were incubated with a streptavidin-Texas red conjugate (12.5 μg/ml, Chemicon) for a further hour. To detect α-SMA reactivity, sections were then incubated with α-SMA (3 μg/ml, DAKO) for 1 h. Reactivity was then determined using a sheep anti-mouse FITC-conjugated antibody (12 μg/ml, Chemicon) for a further 1 h at room temperature. Confocal images were collected using a Bio-Rad confocal inverted Nikon Diaphot 300 microscope (Bio-Rad; Hampstead, UK) equipped with an air-cooled 25-mW argon/krypton laser with wavelengths at 488, 586, and 647 nm and triple dichronic and 568-nm filter sets. Digital images were produced from an average of eight scans of 1-s duration with a ×40 oil-immersion lens and collected using Bio-Rad Laser-Sharp software.

Computer-assisted morphometric analysis. Image analysis was used to quantify α-SMA-positive HSCs. Twenty consecutive nonoverlapping fields at ×100 magnification were analyzed by image-analysis software (Scion Image for Windows, version 4.0.2, National Institutes of Health) to assess the immunofluorescent area per biopsy. For each section, the positive area for replicate fields was summed.

HSC isolation and culture. HSCs were isolated from normal Wistar rats by sequential pronase and collagenase perfusion as previously described (30). Cells were separated on a two-step discontinuous gradient of nycodenz (Sigma; Sydney, Australia), and purity was assessed by vitamin A autofluorescence and flow cytometry (9). The presence of other contaminating nonparenchymal cells (e.g., Kupffer cells and endothelial cells) was also assessed by flow cytometry. After

Trypan blue exclusion to determine viability, 1×10^6 cells/ml were cultured in M199 supplemented with 10% fetal bovine serum and 10% normal horse serum (Trace Biochemicals; Noble Park, Australia) on petri dishes to allow culture activation. Media were changed after 24 h and every 48 h thereafter. Culture-activated cells were routinely used between *passages 6* and *9*.

HSC proliferation. Proliferation of freshly isolated and activated HSCs was assessed using an in-house assay. Briefly, 0.5×10^5 (freshly isolated) or 1×10^4 (culture-activated) cells were seeded onto 24-well plates overnight at 37°C. Cell culture medium was aspirated and replaced with M199 – 0.1% BSA for 18 h. Cells were exposed to various concentrations of rh-FS288 for 18 h and pulsed with 0.5 μCi [³H]thymidine for 16 h. Both adherent and nonadherent cells were harvested after trypsinization and analyzed.

HSC collagen production. Collagen secreted into the cell culture medium was measured using the Sircol Sirius red dye colorimetric assay (Biocolar; Newtown Abbey, Northern Ireland) as previously described (38).

Western blot analysis. Cultured HSCs were washed twice with PBS, suspended in Laemmli buffer, and heated to 95°C for 5 min. After centrifugation, the supernatant was collected, and the protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce). Twenty micrograms of protein were separated by SDS-PAGE (10% gradient gel) under reduced conditions and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was then blocked overnight at 4°C in PBS-0.1% Tween 20-5% skim milk powder and incubated for 1 h at room temperature with primary antibody (α-SMA, DAKO, 1:5,000). The membrane was then stripped and reprobed with β-tubulin (Sigma, 1:5,000) as a loading control. After being incubated with peroxidase-labeled secondary antibody (goat anti-mouse horseradish peroxidase conjugate, Pierce, 1:10,000) for 1 h at room temperature, the membrane was washed in PBS-0.1% Tween 20 and exposed to X-ray film using ECL Western blotting detection reagent (Pierce).

RNA purification and reverse transcription. Total RNA was purified using TRIzol (Invitrogen) with modifications. Briefly, after the initial extraction, supernatants were mixed with a high-salt solution of 0.8 M sodium citrate and 1.2 M sodium chloride to allow more efficient precipitation. RNA concentration was quantitated by 260-to-280-nm absorbance spectrophotometry. For each sample, 2 μg RNA was transcribed using Superscript II (Promega).

Real-time PCR analysis. mRNA analysis was performed using the Roche LightCycler (Roche Biochemicals; Branchbery, NJ). Normal rat cDNA was the control in all reactions to ensure that cycling conditions remained constant. The expression level of each mRNA and the estimated crossing points of each sample were determined using the software provided. A ratio of specific mRNA target relative to the housekeeping gene GAPDH was calculated. Reagents were obtained from Roche Biochemicals. For PCR, 2 μl of each cDNA preparation were diluted to a final concentration of 1:400 (see Table 1 for primers). Forty cycles of PCR were used to ensure that a log-linear phase was reached. At the completion of each reaction, melting curve analysis was performed to establish specificity of DNA products. In every instance, each primer set for individual animals was performed in a single PCR experiment. The intra-assay variation was 4% for each primer set.

Statistical analyses. Each experiment was repeated at least three times. Statistical analyses were performed using ANOVA with comparisons between groups assessed using the Student-Newman-Keuls test. Nonparametric data were analyzed by the Mann Whitney *U*-test. *P* values of <0.05 were considered significant. All analyses were conducted using GraphPad Prism 3 software.

RESULTS

Establishment of cirrhosis. CCl_4-treated animals showed expanded portal tracts by *week 2* compared with the normal

Table 1. *Forward and reverse primer sequences used for real-time PCR analysis for nominated genes*

	Sequence
Activin-β_A	
Forward	5′-TCGAGTGTGATGGCAAGGTC-3′
Reverse	5′-TGTCAGGCGCAGCCACACTCCT-3′
Follistatin	
Forward	5′-GGGCAGGATCCATTGGATTACCCTAT-3′
Reverse	5′-ACACTGCTGGACAGTTTACCACTCT-3′
TGF-β_1	
Forward	5′-CTCGGGGGCTGCGGCTACTG-3′
Reverse	5′-GGCGTATCAGTCGGGGGTCA-3′
Type I collagen	
Forward	5′-ATGTTCAGCTTTGTGGACCT-3′
Reverse	5′-CAGCTGACTTCAGGGATGT-3′
GAPDH	
Forward	5′-GACCCCTTCATTGACCTCAAC-3′
Reverse	5′-GATGACCTTGCCCACAGCCTT-3′

TGF-β_1, transforming growth factor-β_1.

liver (Fig. 1A) and, by *week 8*, most (95%) animals showed bridging fibrosis with some animals exhibiting definite cirrhosis (Fig. 1B).

Cellular expression of activin but not follistatin is modified in the fibrotic liver. Activin A immunoreactivity in the normal liver localized to hepatocyte cytoplasm and was evenly distributed throughout the lobule (Fig. 1C). By *week 4*, activin A was primarily localized to periscar hepatocytes (Fig. 1D) with reduced immunoreactivity in lobular hepatocytes. Activin A immunoreactivity colocalized with α-SMA within fibrotic bands, implicating activated HSCs as an activin source within the injured liver (Fig. 2).

Comparable follistatin immunolocalization studies have not been previously reported. We found that diffuse staining for follistatin was evenly distributed in hepatocyte cytoplasm (Fig. 1E) and, in contrast to activin A, did not redistribute to fibrotic septae (Fig. 1F).

Activin, but not follistatin, gene expression is upregulated in HSCs. To investigate the apparent dysregulation of activin A and follistatin highlighted by the immunohistochemical analyses, HSCs were isolated from the normal liver and activated in vitro. Typically, isolated cell populations were >90% pure HSCs, expressed HSC markers such as desmin, and were negative for von Willebrand factor (endothelial cells) and ED₁ (Kupffer cells). Although freshly isolated HSCs did not express α-SMA, this activation marker was expressed abundantly after 3–5 days in culture (data not shown).

Activin A production increased as HSCs transformed to a myofibroblast phenotype (Fig. 3A) associated with increases in α-SMA expression (Fig. 3, *B* and *C*) and collagen production (Fig. 3D). We next explored changes at the mRNA level. Real-time PCR analysis of activating HSCs revealed that activin, follistatin, TGF-β, and type I collagen mRNA all significantly increased 1 day after culture ($P < 0.01$), but activin mRNA increased proportionally more than other transcripts (Fig. 4). Expression of all mRNAs continued to increase as HSCs activated with the exception of follistatin, which peaked at *day 1* of culture and returned to baseline by *day 5*.

Follistatin inhibits HSC proliferation in vitro. Because proliferating HSCs produced increasing amounts of activin A during activation without a concurrent increase in follistatin

gene expression, we analyzed the effect of exogenous follistatin on proliferation of freshly isolated and activated HSCs. We observed a moderate dose-dependent decrease in the proliferation of both freshly isolated and culture-activated HSCs (Fig.
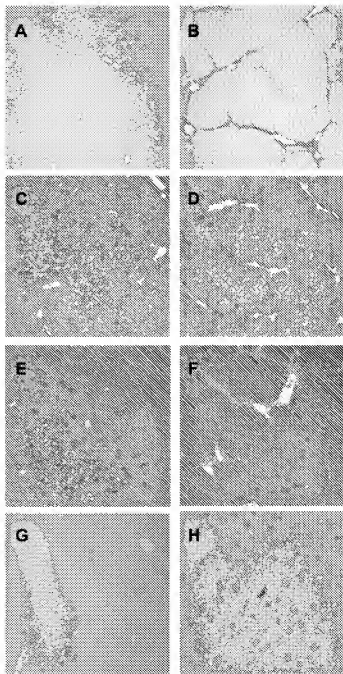

Fig. 1. Immunohistochemical localization of activin A and follistatin in normal and cirrhotic livers. *A*: normal tissue displayed no abnormal fibrosis (Sirius red staining). *B*: most rats treated with CCl₄ had probable or definite cirrhosis by *week 8*. *C*: activin A immunoreactivity in the normal liver was diffuse, lobular, and restricted to the cytoplasm of hepatocytes. *D*: in the fibrotic liver, activin A reactivity was localized to fibrous septae. *E*: follistatin immunolocalization had a similar pattern to that of activin A in the normal liver, being diffuse and lobular, which was not observed to change in the fibrotic liver (*F*). *G*: negative control for activin using an inappropriate antibody; no reactivity for activin A was observed. *H*: apoptotic hepatocytes were visualized using TdT-mediated dUTP nick-end labeling as outlined in METHODS. Original magnification: ×50 in *A–G* and ×200 in *H*.
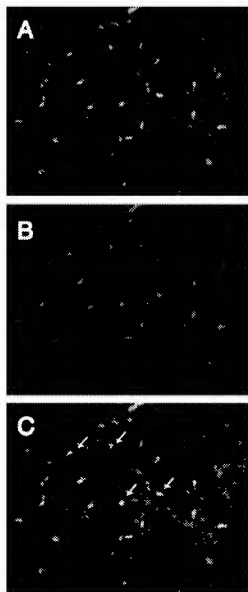
Fig. 2. Co-localization of activin with α-smooth muscle actin (α-SMA). Liver sections from animals receiving 2 wk of CCl₄ showed hepatic stellate cell (HSC) staining for α-SMA (*A*; green), a HSC activation marker, and activin A (*B*; red). *C*: confocal microscopy showed double staining (yellow), confirming that activated HSCs express activin. White arrows indicate representative colocalization. Control staining of identical sections with inappropriate primary antibody was invariably negative (not shown). Original magnification: ×50.

5*A*) that was greater in freshly isolated HSCs compared with cells with an activated myofibroblast-like phenotype (Fig. 5*B*).

Attenuation of hepatic fibrogenesis by exogenous follistatin. Because the above observations supported a role for dysregulation of the activin-follistatin axis during hepatic fibrogenesis, we then administered follistatin during CCl₄-induced hepatic fibrosis. Follistatin-treated animals had significantly lower hepatic hydroxyproline content 2 and 4 wk after CCl₄ treatment, reaching a maximal decrease of 32% at *week 2*. This trend continued to *week 8* (Fig. 6). Histological fibrosis was clearly attenuated in follistatin-treated animals at early time points, although by *week 8* most (70%) animals demonstrated establishment of fibrosis (Fig. 7). Quantification of activated HSCs
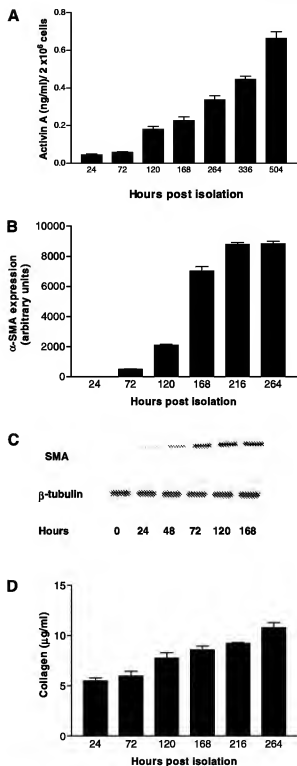

Fig. 3. Isolated HSCs produce increasing quantities of activin A as they transform to a myofibroblast phenotype. *A*: freshly isolated HSCs were cultured in standard conditions, and conditioned medium was analyzed for activin A by ELISA. *B*: expression of α-SMA by cultured rat HSCs was measured by densitometry, and the ratio of α-SMA to β-tubulin was determined. Values are expressed in arbitrary units. *C*: representative Western blot of α-SMA expression by freshly isolated cultured rat HSCs. β-Tubulin was measured as an internal control. *D*: collagen production was measured in freshly isolated cultured rat HSCs using a colorimetric assay as outlined in METHODS. All data are means ± SE of 3 separate HSC isolates.
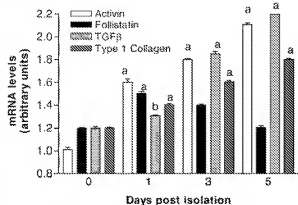
Fig. 4. Gene expression by real-time PCR in cultured HSCs. Activin subunit mRNA levels increase rapidly during HSC transformation to a myofibroblast phenotype in culture, proportionally more than either transforming growth factor (TGF)-β_1 or type I collagen mRNAs. Follistatin mRNA levels showed a transient increase on *day 1* of culture and then declined. Levels of all transcripts are expressed relative to GAPDH mRNA expression. [a]$P < 0.01$ compared with the *day 0* value; [b]$P < 0.05$, activin compared with either TGF-β_1 or type I collagen at the *day 1* time point. All data are expressed as means $\pm$ SE and are representative of 3 separate experiments.

was consistent and revealed significantly lower numbers of activated HSC at *weeks 2* and *8* in follistatin-treated animals compared with untreated controls (Fig. 8).

Reduction of hepatocyte apoptosis. Apoptotic events may trigger inflammation and fibrosis (2), and, because activin is a potent inducer of hepatocyte apoptosis, we compared the extent of hepatocyte apoptosis in follistatin-treated animals with untreated controls. We found that hepatocyte apoptosis was significantly decreased by follistatin treatment at all time points and maximal by 87% at *week 4* (Fig. 9). A typical example of hepatocyte apoptosis is shown in Fig. 1G.

DISCUSSION

Activation of quiescent HSCs to a profibrogenic phenotype is a critical event in the pathogenesis of hepatic fibrosis, and TGF-β is the prototypical driver of this process. We investigated the role of activin A, a member of the TGF-β superfamily, in HSC activation and fibrogenic activity. Activin is a cytokine that regulates hepatic homeostasis and mass (21), an effect antagonized by follistatin, the major activin binding protein. This interaction between activin A and follistatin is typical of most biological systems in which expression of the two molecules is tightly controlled with circulating activin irreversibly bound to follistatin and neutralized (32), such that a biological effect occurs only when activin is unopposed by follistatin (27). Previous reports (5, 34) have shown that activin A expression is upregulated in hepatic fibrosis. In this study, we extended these findings by investigating activin A expression during HSC activation and by determining whether inhibition of activin by exogenous follistatin administration could ameliorate activin-induced hepatic fibrosis. We found that activin, but not follistatin, gene expression is upregulated in isolated rat HSCs and that HSCs secrete activin in significant quantities during myofibroblast transformation. Furthermore, analysis of activin expression in an animal model of CCl$_4$-induced liver injury revealed that activin A localized to areas of periscar hepatocytes and activated HSCs.
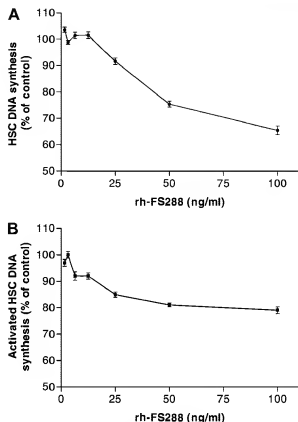

Fig. 5. The addition of recombinant human follistatin-288 (rh-FS288) to cultures of either freshly isolated HSCs (A) or HSCs that had been in culture for 7 or more days and were considered activated (B) resulted in a dose-dependent decrease in cellular DNA synthesis as determined by [^{3}H]thymidine incorporation. All data are expressed as means $\pm$ SE and are representative of 3 separate experiments.

The demonstration that exogenous follistatin attenuated early fibrogenic events further supports the biological significance of activin A during the development of hepatic fibrosis.

A striking and consistent feature in this and other reports (5, 34) has been the redistribution of activin A immunoreactivity
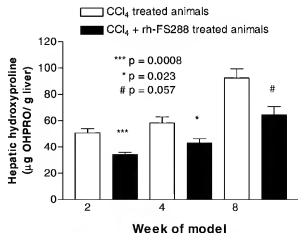

Fig. 6. The administration of rh-FS288 to CCl$_4$-treated animals resulted in a significant decrease in hepatic hydroxyproline content at *week 2* ($P = 0.0008$) and *week 4* ($P = 0.023$) compared with controls given CCl$_4$ alone. A similar trend was apparent at *week 8* ($P = 0.057$).
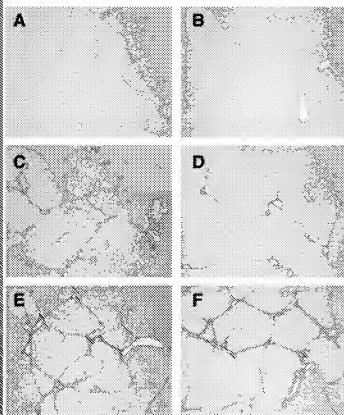
Fig. 7. Histological assessment by Sirius red staining of liver sections from animals injected with rh-FS288 and CCl₄ revealed significant improvement in fibrosis compared with control animals at *week 2* [control (*A*) vs. rh-FS288 treated (*B*)] and *week 4* (*C* vs. *D*). However, by *week 8*, most animals injected with rh-FS288 displayed a similar stage of fibrosis compared with controls (*E* vs. *F*).

Fig. 8. Quantitative computer-assisted morphometric analysis of immunofluorescent staining of activated HSCs (α-SMA-positive cells) in follistatin-treated animals compared with controls. Analysis revealed significantly fewer α-SMA-positive cells in rh-FS288-treated animals compared with controls at 2 and 8 wk ($P = 0.04$).

from the hepatic lobule to fibrous septae. We have now shown for the first time that this redistribution is unaccompanied by changes in follistatin localization. In contrast to Sugiyama and colleagues (34), who were unable to demonstrate activin A immunoreactivity in the normal liver, we detected significant hepatocyte staining. The differences in activin A immunolocalisation may reflect different antibodies for the activin β$_A$-subunit used in each study. The E4 monoclonal antibody used for immunohistochemical localization in this study has been extensively validated (17, 23, 24). We also observed increased hepatocyte apoptosis in the fibrotic liver, although without a specific cellular distribution pattern, and no correlation was detected between activin A immunoreactivity and the septal location of apoptotic hepatocytes, as reported previously (25).

We found that during culture activation, HSCs secreted activin A protein due to increased activin mRNA expression and that follistatin mRNA expression did not increase above basal levels, consistent with the finding that HSC-derived activin was biologically active and unopposed by follistatin. In the fibrotic liver, α-SMA-positive HSCs strongly expressed activin immunoreactivity. HSC-derived activin may have a number of local consequences, consistent with the concept that activin and follistatin act primarily in a paracrine or autocrine manner (37). Previous studies have reported that activin may stimulate the production of matrix proteins such as collagen (36) and fibronectin (4), which are produced early in HSC
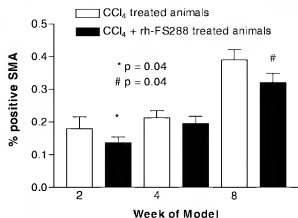
activation (1). Furthermore, activin A increases the expression of type I collagen mRNA in HSC cultures either alone or in synergy with TGF-β (34). We found that activin mRNA expression in HSCs increased earlier than that of type I collagen, supporting the view that activin acts in combination with TGF-β to stimulate matrix production. It is also worth noting that, whereas TGF-β is secreted as a pro-protein requiring enzymatic cleavage, activin A is secreted as an active molecule (28), consistent with the view that activin A may be an important early mediator of hepatic fibrosis.

Administration of follistatin, the most potent biological inhibitor of activin, resulted in a significant reduction in hepatic hydroxyproline content and histological fibrosis. Three interrelated mechanisms may explain these findings. First, administration of exogenous follistatin may neutralize activin-induced events. In a recent study, Wada et al. (36) studied the effect of activin A on primary cultures of rat HSCs and demonstrated that activin was able to increase HSC α-SMA expression in vitro. We have extended these findings to an in vivo model by demonstrating significantly fewer activated HSCs within the hepatic lobule in follistatin-treated animals.
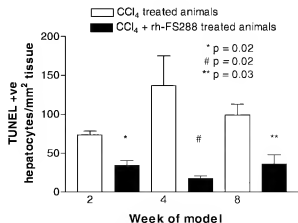

Fig. 9. Administration of rh-FS288 to CCl₄-treated animals significantly reduced hepatocyte apoptosis at *weeks 2, 4* ($P = 0.02$) and *8* ($P = 0.03$) compared with controls given CCl₄ alone.

Thus follistatin binding to HSC-derived activin may diminish autocrine activation of HSCs, and this is consistent with our observation that follistatin administration caused a dose-dependent decrease in HSC proliferation. The concept that follistatin neutralizes activin-induced events is not restricted to the liver. A recent report (6) found that local and systemic upregulation of activin occurred in different models of colonic inflammation and that exogenous follistatin administration significantly improved disease outcome with increased survival of treated animals.

A second potential mechanism for follistatin reduction in hepatic fibrosis may relate to the prevention of hepatocyte apoptosis. Activin has previously been shown to induce hepatocyte apoptosis both in vitro and in vivo when present in excess quantities (12, 33). We found that follistatin administration significantly decreased hepatocyte apoptosis in CCl_4-treated animals. The mechanistic importance of this observation arises from the observation that hepatocyte apoptosis is linked to hepatic fibrosis progression due to cholestasis and alcoholic and nonalcoholic steatohepatitis (2). A recent study (35) has confirmed this important nexus by showing that disruption of the Bcl-X_L gene, an antiapoptotic Bcl-2 family member, caused ongoing hepatocyte apoptosis and the development of progressive hepatic fibrosis. With regard to activin, it has been previously demonstrated that Bcl-X_L upregulation inhibited activin-induced apoptosis in a mouse hybridoma model (22). Thus inhibition of activin-induced hepatocyte apoptosis by follistatin should also contribute to the observed decrease in hepatic fibrosis in this study.

Finally, Date and colleagues (4) have proposed that activin A released from injured hepatocytes interacts with activin receptors present on HSC's to induce fibronectin synthesis. Administration of follistatin may bind activin, preventing interaction with HSC activin receptors and preventing downstream signaling events leading to extracellular matrix synthesis. This mechanism is supported by previous findings that showed decreased HSC collagen synthesis in vitro (36) and by our findings demonstrating decreased deposition of the extracellular matrix in vivo after follistatin administration. Thus several lines of evidence exist to explain how follistatin can inhibit activin A-mediated hepatic fibrogenesis.

We recognize that there is variability among some of the experimental outcomes regarding fibrosis in follistatin-treated animals, such as the morphometric data in Fig. 7, which shows less attenuation of fibrosis at week 8 compared with hydroxyproline content (Fig. 6) and the decrease in the proportion of activated HSCs (Fig. 8). These differences in outcome may reflect the use of a constant dose of follistatin rather than one corrected for body weight in growing animals, or a differential time course for morphological alteration that lags behind the decline in HSC activation and collagen production. However, a more likely explanation lies in the multifactorial nature of hepatic fibrogenesis, which involves multiple mediators such that inhibition of one factor is unlikely to prevent hepatic fibrosis completely. For example, inhibition of TGF-β, the prototypical profibrogenic cytokine, does not completely abrogate fibrosis (10, 14, 29).

In conclusion, activin A expression is markedly altered during the progression of hepatic fibrosis with changes in both the cellular site of expression and in follistatin regulation. Administration of follistatin significantly attenuated early he-

patic fibrogenesis. We propose that follistatin attenuated hepatic fibrosis by reducing activin-induced activation of HSCs and therefore extracellular matrix accumulation in addition to inhibiting activin-mediated hepatocyte apoptosis. Further studies are required to define the relative contribution of these mechanisms to hepatic inflammation and wound repair.

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